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Proliferation Marker pKi-67 Affects the Cell Cycle in a Self-Regulated Manner

Mirko H.H. Schmidt,[1,3] Rainer Broll,[1] Hans-Peter Bruch,[2] and Michael Duchrow[1]*

[1]Surgical Research Laboratory, University of Lübeck, Lübeck, Germany
[2]Department of Surgery, University of Lübeck, Lübeck, Germany
[3]Henry Ford Hospital, Hermelin Brain Tumor Center, Department of Neurosurgery, Detroit, Michigan 48202

Abstract The proliferation marker pKi-67 is commonly used in research and pathology to detect proliferating cells. In a previous work, we found the protein to be associated with regulators of the cell cycle, controlling S-phase progression, as well as entry into and exit from mitosis. Here we investigate whether pKi-67 has a regulative effect on the cell cycle itself. For that purpose we cloned four fragments of pKi-67, together representing nearly the whole protein, and an N-terminal pKi-67 antisense oligonucleotide into a tetracycline inducible gene expression system. The sense fragments were C-terminally modified by addition of either a nuclear localization sequence (NLS) or a STOP codon to address the impact of their intracellular distribution. FACS based cell cycle analysis revealed that expression of nearly all pKi-67 domains and the antisense oligonucleotide led to a decreased amount of cells in S-phase and an increased number of cells in G_2/M- and G_1-phase. Subsequent analysis of the endogenous pKi-67 mRNA and protein levels revealed that the constructs with the most significant impact on the cell cycle were able to silence pKi-67 transcription as well. We conclude from the data that pKi-67 influences progression of S-phase and mitosis in a self-regulated manner and, therefore, effects the cell cycle checkpoints within both phases. Furthermore, we found pKi-67 mediates an anti-apoptotic effect on the cell and we verified that this marker, although it is a potential ribosomal catalyst, is not expressed in differentiated tissues with a high transcriptional activity. J. Cell. Biochem. 87: 334–341, 2002. © 2002 Wiley-Liss, Inc.

Key words: pKi-67; cell cycle regulation; S-phase; mitosis; apoptosis

The proliferation marker pKi-67 was originally described by Gerdes et al. [1983] as a nuclear protein associated with the active phases of the cell cycle (G_1-, S-, G_2-, and M-phase), but not detectable in quiescent cells (G_0-phase) [Gerdes et al., 1984]. Because of this restricted expression, the pKi-67 specific antibodies Ki-67 and MIB-1 became commonly used proliferation detection reagents. In detail, pKi-67 is localized within the dense fibrillar components of the nucleoli from early G_1-phase until middle G_2-phase [Kill, 1996]. At the G_2 to M transition, while the nucleoli disintegrate, pKi-67 spreads throughout the nucleoplasm and forms a mesh-like structure that is weakly associated with the condensing chromosomes [Verheijen et al., 1989; Ross and Hall, 1995; Scholzen and Gerdes, 2000]. During the progression of mitosis it becomes part of the perichromosomal scaffold, surrounding the chromosomes like a glove [Gautier et al., 1992a,b,c]. By the end of mitosis the chromosomes decondense and the scaffold disappears. The pKi-67 protein itself occurs in two isoforms with approximate molecular weights of 395 and 345 kDa encoded by the same gene. The shorter version of pKi-67 results from an alternative splicing event of exon 7 during mRNA processing. Cloning of the *pKi-67* cDNA revealed a large mRNA (about 12,500 bp) with 15 exons [Schluter et al., 1993; Duchrow et al., 1996]. Exon 13 encodes the core of the protein, containing the 16 tandem repeats recognized by most of the commercially available antibodies. pKi-67 binds DNA [MacCallum and Hall, 2000b], it contains an ATP/GTP-binding

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*Correspondence to: Michael Duchrow, Surgical Research Laboratory, Department of Surgery, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.
E-mail: duchrow@medinf.mu-luebeck.de

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site at the C-terminus [Schluter et al., 1993; Duchrow et al., 1994, 1995], as well as a large number of phosphorylation sites [Heidebrecht et al., 1996], which are targeted to yield a high phosphorylation status of the protein during the cell cycle [MacCallum and Hall, 1999; Endl and Gerdes, 2000].

Using a two-hybrid system, we recently found pKi-67 to be associated with the DNA-synthesis regulators MCM2 and p95 [Schmidt et al., 2002], which function during S-phase. We showed in the same work that pKi-67 can influence the disruption and reformation of the nucleoli, possibly via a functional relationship with cyclin B and the small GTPase Ran, and, therefore, regulates the cell cycle. To study the effects more closely, we cloned four fragments of pKi-67, together representing nearly the whole protein, and an N-terminal pKi-67 antisense oligonucleotide into a tetracycline inducible gene expression system. Subsequently, we analyzed the influence of these fragments on the cell cycle using FACS analysis. Moreover, MacCallum and Hall [2000b] showed that overexpression of the C-terminus leads to the detachment of the chromosomes from the inner side of the nuclear lamina and thus to apoptosis. Based on this observation, we used our expression constructs to analyze the effects of the recombinant pKi-67 fragments on programmed cell death. Finally, van Oijen et al. [1998] showed that pKi-67 is expressed in cell cycle arrested cells. In light of our finding that pKi-67 is involved in the lifecycle of the nucleoli [Schmidt et al., 2002], the ribosome production machinery, and the proposal that pKi-67 might act as a ribosome production catalyst [MacCallum and Hall, 2000a], it seemed possible that the protein is transcribed in differentiated cells with a high rate of protein synthesis. To address this question we screened several glandular and neuronal cells with MIB-1 and found these cells to be pKi-67 negative.

MATERIALS AND METHODS

Cells Lines and Cell Transfection

HT-OFF cells (HeLa cells carrying the regulator plasmid pTet-OFF of the Tet-system) were cultured under standard conditions in DMEM supplemented with antibiotics and 10% FCS. Cells were transiently transfected using QIAGEN Polyfect™ reagent (QIAGEN, Hilden, Germany) and harvested 48 h after transfection. Generally, experiments were repeated independently at least three times.

Tet-OFF System™

This tetracycline gene expression system [Gossen and Bujard, 1992, 1993, 1995; Gossen et al., 1993, 1994, 1995] was obtained from Clontech and used according to the manufacturers guidelines. *pKi-67* fragments (Fig. 1A) were amplified with *Pfu* proofreading DNA polymerase (Promega, Mannheim, Germany) from HeLa cDNA, cloned into the expression vector pUHD 10/3 (now known as pTRE), and were sequenced as a control. The recombinant *pKi-67* fragments were modified by addition of a Kozak sequence at the 5′ end as well as either a STOP codon or a nuclear localization sequence (NLS) before the STOP codon at the 3′ end. Recombinant protein expression was blocked by addition of 5 ng/ml doxycycline to the cell culture medium. Correct protein expression was controlled by mRNA analysis (Fig. 1B) and immunoblotting with MIB-1, MIB-7, MIB-21, and MIB-24 (Dako, Glostrup, Denmark; Fig. 1C). The antisense construct AS was not

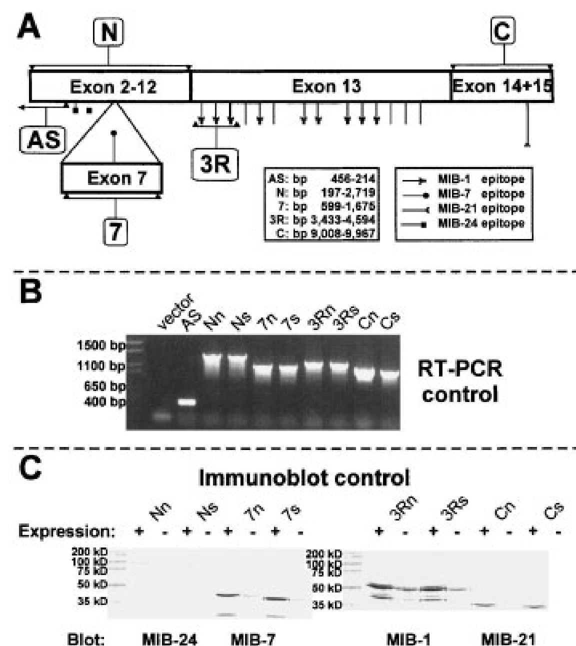

Fig. 1. pKi-67 domains cloned into the Tet-OFF system. **A**: Illustration of the *pKi-67* fragments used in this study. (AS, N-terminal antisense oligonucleotide; N, N-terminus; 7, exon 7; 3R, repeats one to three of exon 13; C, C-terminus). The regions they cover in the GenBank sequence file NM_002417 are indicated in base pairs [Schluter et al., 1993]. **B**: RT-PCR and (**C**) immunoblot controls of the expression vectors used in this work.

examined in westerns, since it was not supposed to produce a protein.

Immunoblotting and Immunohistochemistry

Endogenous pKi-67 protein was isolated using a modified protocol of MacCallum and Hall [1999]. Recombinant pKi-67 fragment carrying cells were simply lysed in Laemmli buffer at 95°C. Following standard SDS–PAGE [Laemmli, 1970] proteins were blotted on a PVDF membrane. As a primary antibody, MIB-1 was used at a concentration of 1 μg/ml, all other MIB antibodies at a concentration of 40 μg/ml. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (A-2429; Sigma, Deisenhofen, Germany) was used as a secondary antibody in a dilution of 1:5,000 in blocking buffer and visualized with BCIP/NBT as the chromogen mixture in a concentration of 9 μg/ml each.

pKi-67 specific immunohistochemical staining was performed with MIB-1 (Dako) as previously described by Cattoretti et al. [1992] with modifications. In brief, pancreas and colon tissues were fixed in 4.5% formalin and embedded into paraffin as surgical pathology specimens in a routine manner. Four micrometer serial sections were de-waxed in xylene, rehydrated in graded alcohol, and pre-treated for antigen retrieval in citrate buffer (pH 6) in a microwave-oven (800 W) for four cycles of 10 min. Bone marrow samples were spread out on slides, were air dried for 30 min and afterwards frozen at −80°C. Plasma cells were identified by co-staining with mAb CD138 (Dako). Tissue preparations were stained with MIB-1 (final concentration 1 μg/ml) using the HRP conjugated streptavidin–biotin complex (ABC-Staining from Dako) as a secondary reagent and DAB as a chromogen.

Flow Cytometry and Detection of Apoptotic Cells

DNA staining of transfected cells was carried out using the Cycle Test Plus DNA Reagent Kit (BD, Heidelberg, Germany). In brief, nuclei of transfected cells (5×10^5/500 μl) were isolated by disruption of the cell membrane and the cytoskeleton with a combination of trypsin and spermine tetra-hydrochloride. DNA was stained with propidium iodide (PI) at a final concentration of at least 1.25 μg/ml. Nuclei were analyzed with a FACScan (BD), fitted with an Argon ion laser adjusted to emit 500 mW at 480 nm and a doublet discriminator. Red fluorescence (PI) was measured through an Lp 650 nm filter. Fluorescence distributions were analyzed with the computer programs Cellquest and ModFit (supplied by BD).

To detect apoptosis, cells were stained with a FITC conjugated rabbit anti-PARP cleavage site (214/215) specific polyclonal antibody (Biosource, Sollingen, Germany) according to the manufacturers guidelines. Within 1 h fluorescence was detected and analyzed with the FACScan device as described above.

RESULTS

pKi-67 fragments were cloned into the Tet expression vector as shown in Figure 1A. The recombinant cDNA sequences were modified by addition of a Kozak sequence at the 5′ end as well as either a STOP codon or a NLS before the STOP codon at the 3′ end. The resulting plasmids were transiently transfected in HT-OFF cells, which were harvested after 48 h. Recombinant protein expression was blocked by addition of 5 ng/ml doxycycline to the cell culture medium. In order to analyze the cellular effects of recombinant pKi-67 fragments with and without NLS, we determined the distribution of cells within different phases of the cell cycle with (+) and without (−) transcription of the, respectively, transfected tetracycline regulated plasmid. A representative experiment is shown in Figure 2. Mainly S-phase was affected by the expression of recombinant pKi-67 fragments. All constructs, including the antisense oligonucleotide (AS), caused a decrease in the amount of cells within this phase although to different extents. The reduction of cells in S-phase was usually accompanied by an increased amount of cells in both the G_2/M- and the G_1/G_0-fraction, but not after expression of the C-terminus of pKi-67 without NLS. In this case, we found a complete shift of cells from S-phase to G_1-phase. This indicates that this partial structure of pKi-67 effects the cell cycle slightly different from the other constructs.

To investigate the influence of the recombinant pKi-67 domains on endogenous pKi-67, we transiently transfected HT-OFF cells with our constructs and harvested them after 48 h. Transcription was blocked by addition of 5 ng/ml doxycyclin to obtain control cells. We split each sample and used one part to isolate the mRNA, the other to isolate endogenous pKi-67 protein.

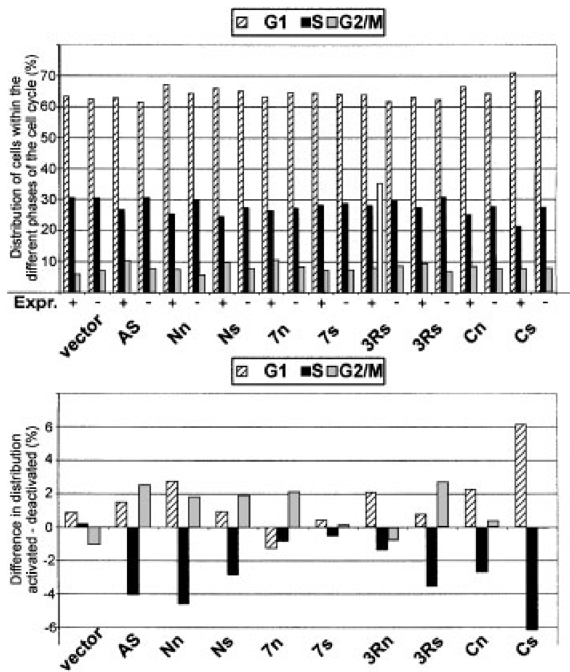

Fig. 2. Cell cycle analysis of transiently transfected HeLa cells. Influence of recombinant pKi-67 fragments on the cell cycle. The upper diagram shows the distribution of cells within the different phases of the cell cycle. The lower diagram illustrates the differences between plasmid transcribing (+) and non-transcribing (−) cells (transcription inhibited by addition of 5 ng/ml doxycycline). AS, N-terminal antisense oligonucleotide; N, N-terminus; 7, exon 7; 3R, repeats one to three of exon 13; C, C-terminus; n, 3′-terminal NLS; s, 3′-terminal STOP codon.

MRNA was reverse transcribed and PCR with endogenous pKi-67 specific primers were performed. To monitor the expression of endogenous pKi-67 in cells transfected with Nn, Ns, 7n, 7s, 3Rn, 3Rs as well as the antisense oligonucleotide (AS), a primer pair targeted against the C-terminus of *pKi-67* (bp 9,008–9,963) was used. In samples transfected with recombinant Cn and Cs, we amplified a repeat region in exon 13 (3,433–3,960). Hereby, we ensured the detection of endogenous *pKi-67* only and avoided misinterpretation of our results due to the transfected plasmids. As a positive control, we amplified β_2-microglobulin that is present in all human cells. Normalizing the *pKi-67* band intensity to this control thereby allowed us to compare the pKi-67 expression in transcription activated and doxycyclin deactivated cells. Amplification of endogenous *pKi-67* (Fig. 3) revealed, that the N-terminal antisense oligonucleotide diminished the amount of its mRNA, while Nn, Ns, 3Rs, and Cn completely blocked pKi-67 transcription. Following SDS–PAGE, this effect was verified at the protein level because the four fragments that diminished pKi-67 mRNA caused a decrease of endogenous pKi-67 protein, too. These fragments also significantly affected the cell cycle (Fig. 2). At the same time 7n, 7s, and 3Rs had little or no effect, neither on pKi-67 mRNA nor on its protein concentration, such as these fragments had only a little effect on the cell cycle (Fig. 2). The correspondence of the effects of the different fragments on the cell cycle and the pKi-67 mRNA/protein suggests a functional relationship between these events.

The influence of pKi-67 on the cell cycle suggested it might have an effect on apoptosis, since

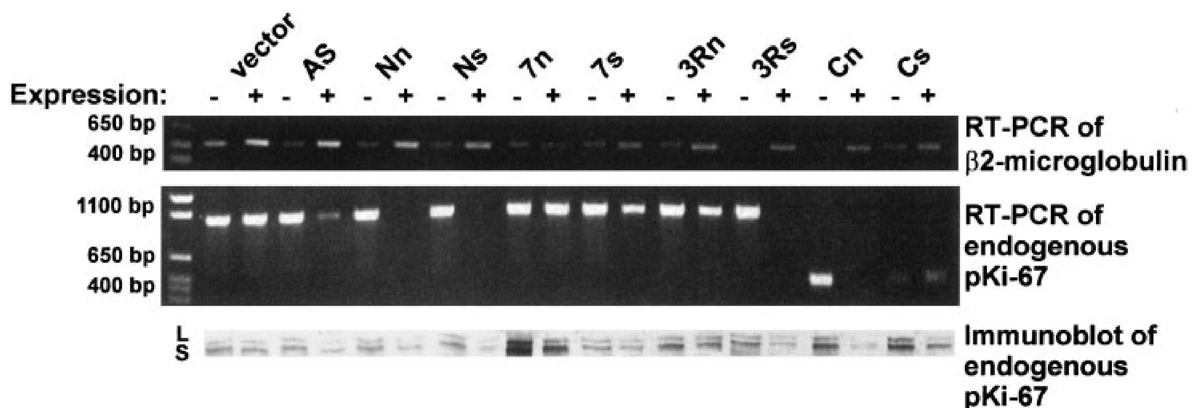

Fig. 3. Impact of transiently transfected *pKi-67* fragments on endogenous *pKi-67*. Cells were transfected with the recombinant Tet-constructs, cultured for 48 h and each dish was split up into two parts. One was used for RT-PCR (upper and middle row), one for immunoblotting with MIB-1 (lower row). The positive control β_2-microglobulin was used to normalize the results. Endogenous *pKi-67* was down-regulated in RT-PCR after expression (+) of AS, Nn and s, 3Rs and Cn compared to the deactivated (−) cells (addition of 5 ng/ml doxycycline). Down-regulation was verified by western (L, large pKi-67 isoform; S, small pKi-67 isoform). AS, N-terminal antisense oligonucleotide; N, N-terminus; 7, exon 7; 3R, repeats one to three of exon 13; C, C-terminus; n, 3′-terminal NLS; s, 3′-terminal STOP codon.

programmed cell death usually depends on the re-entry into the cell cycle [Coates et al., 1996]. For that reason, we stained pKi-67 domain transiently transfected cells with a FITC-conjugated anti-PARP cleavage site specific antibody. Poly (ADP-Ribose) polymerase (PARP) is a 116 kDa nuclear protein, which is strongly activated by DNA strand breaks [Kaufmann et al., 1993; Duriez and Shah, 1997; Germain et al., 1999]. During apoptosis caspases, such as caspase-3 and -7, cleave PARP to yield an 85 and a 25 kDa fragment. PARP cleavage is considered to be one of the classical characteristics of apoptosis. The antibody used here specifically recognized the 85 kDa fragment of cleaved PARP and can, therefore, be used as a marker for apoptotic cells (Fig. 4). Addition of doxycyclin to block transcription of the transfected plasmids caused apoptosis itself. Transcription of most pKi-67 fragments (except 7n) diminished the amount of apoptotic cells and, therefore, seemed to mediate an anti-apoptotic effect. Since the amount of apoptosis reduction is quite low, we are in the opinion that this might be a side-effect of pKi-67's influence on S-phase described in Figure 2.

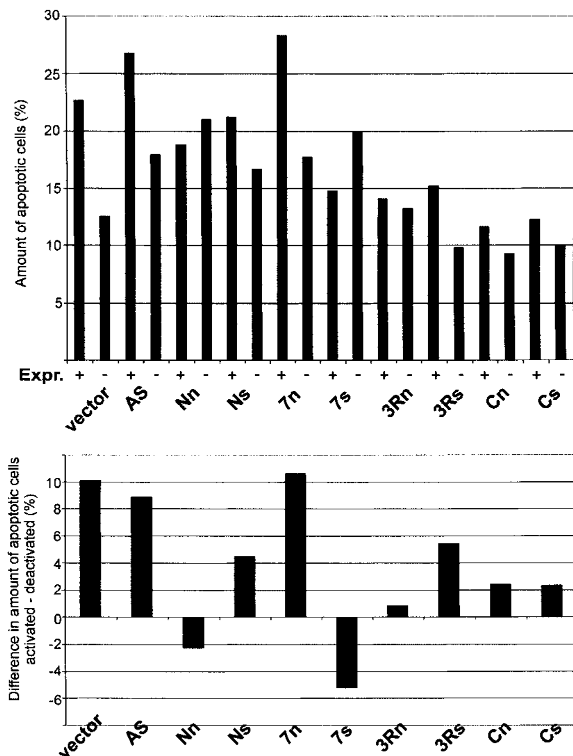

Fig. 4. Amount of apoptosis in transiently transfected HeLa cells. Influence of recombinant pKi-67 fragments on apoptosis. The upper diagram shows the percentage of apoptotic cells in each sample. The lower diagram illustrates differences between plasmid transcribing (+) and non-transcribing (–) cells (transcription inhibited by addition of 5 ng/ml doxycycline). AS, N-terminal antisense oligonucleotide; N, N-terminus; 7, exon 7; 3R, repeats one to three of exon 13; C, C-terminus; n, 3′-terminal NLS; s, 3′-terminal STOP codon.

Finally, we addressed the question how restricted the expression of pKi-67 is to the cell cycle. To test the hypothesis that pKi-67 could be expressed as a ribosomal catalyst in cells with a high protein synthesis rate, we stained differentiated tissues, known to be highly secretory, with MIB-1 (Fig. 5). We used pancreatic tissue, identified the exocrine and endocrine glands and found the nuclei within these areas unstained (Fig. 5A). In colon tissue, we identified the goblet cells and the myenteric plexi, and also found the nuclei of these cells unstained (Fig. 5B,D). The faint dark color of the peripheral nerve cells corresponds to the natural color of the nerve. Finally, we identified antibody secreting B cells in bone marrow with the plasma cell marker CD138 and double-stained them with MIB-1. Again, the nuclei were MIB-1 negative. Summarizing, we found no evidence for an expression of pKi-67 in differentiated, non-proliferating tissue.

DISCUSSION

In this work, we intended to investigate, if recombinant domains of the proliferation marker pKi-67 have direct effects on the cell cycle. For that purpose we cloned the respective pKi-67 fragments into a tetracycline regulated gene expression system. FACS based cell cycle analysis revealed that the expression of most constructs diminished the amount of cells in S-phase. Our findings are consistent with a previous work of us [Duchrow et al., 2001], where we found that overexpression of a pKi-67 antisense construct and recombinant tandem repeats resulted in the reduction of cells in S-phase. N- and C-terminus seemed to be more effective then exon 7. The recombinant tandem repeats (3R) significantly affected the cell cycle only, when not carrying a NLS and, therefore, being restricted to the cytoplasm. Additionally to the effect on cells in S-phase, the C-terminus of pKi-67 strongly increased the amount of cells in G_1-phase. We know from our work [Schmidt et al., 2002] and from a publication by Scholzen et al. [2002] that the C-terminus of pKi-67 binds to the HP1 heterochromatin binding proteins, which support the attachment of the

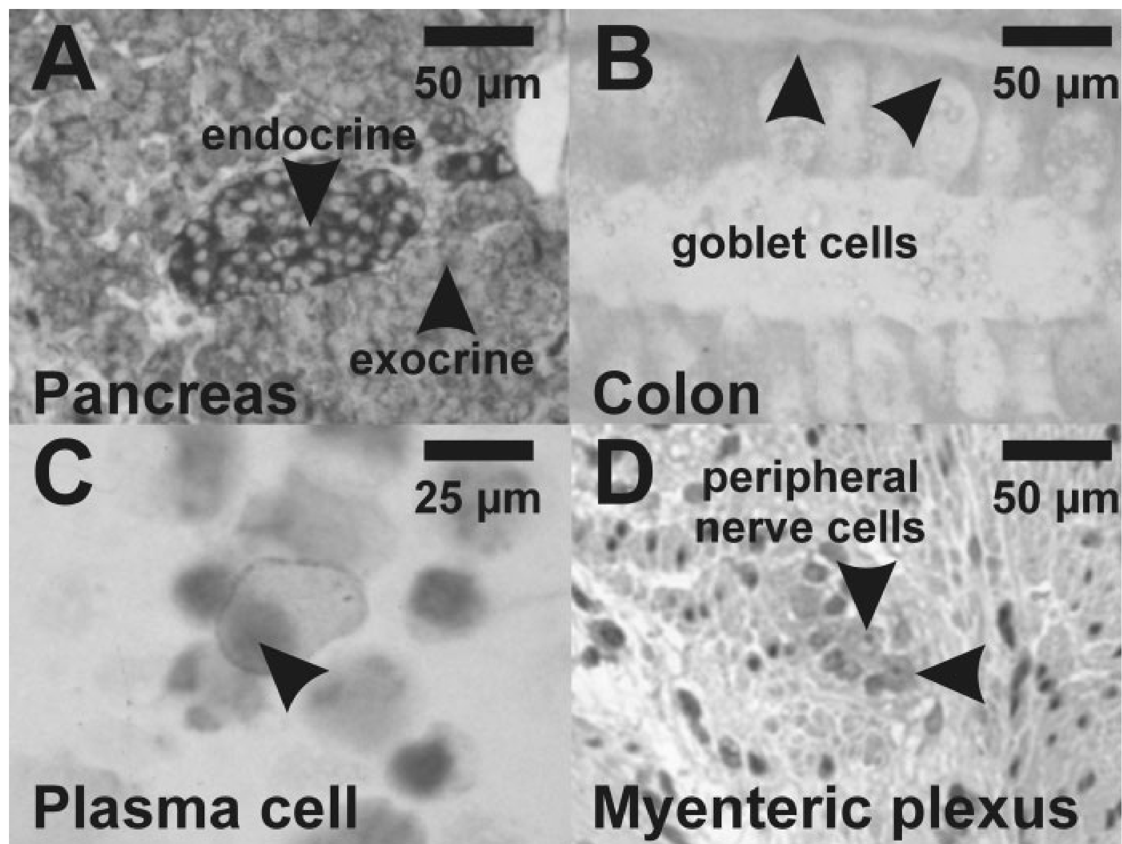

Fig. 5. MIB-1 staining of differentiated tissues with a high protein synthesis activity. Arrows mark unstained nuclei of the respective tissue cells. pKi-67 was not detected in neither of these tissues and is, therefore, not expressed as a ribosomal catalyst in differentiated cells.

chromosomes to the inner side of the nuclear lamina at the end of mitosis. For that reason, we expected this domain to have additional effects compared to the other constructs. We speculate that the C-terminus not only causes a faster passage through S-phase but also a faster exit from mitosis. However, it cannot be ruled out by this experiment that the pKi-67 C-terminus causes a block within G_1-phase.

The use of a transient transfection system resulted in smaller effects on the different cell cycle phases as it might really be, since we measured non-transfected cells as well. We tried to overcome this shortcoming by creating cell lines permanently expressing our constructs in a Tet-ON system (data not shown). Gene expression is induced in this system by addition of tetracycline, not blocked as in the Tet-OFF system we used in this work. It turned out that all cell clones we created used to produce tiny amounts of pKi-67 constructs even in the absence of tetracycline. This "leakiness" resulted in a very different behavior of the cells towards pKi-67 overexpression. The effects on the cell cycle, which were very strong during the very first cell passages, used to decrease over time and eventually vanished after multiple passages of the cells. For that reason, we decided to perform the cell cycle analysis experiments in a transient system, which showed quantitatively smaller but more reproducible results. From our data, we hypothesize that expression of partial pKi-67 structures hampers the function of endogenous pKi-67. This is supported by the observation that the effects on the cell cycle are very similar to those obtained by *pKi-67* antisense oligonucleotide expression. Additionally, RT-PCR of endogenous *pKi-67* revealed that the N-terminal antisense oligonucleotide diminished the amount of *pKi-67* mRNA, while Nn, Ns, 3Rs, and Cn completely blocked pKi-67 transcription. The downregulation was verified on the protein level by immunoblotting with MIB-1. Out of all our constructs these four recombinant pKi-67 domains showed the strongest effect on S-phase as

well (Fig. 3). This indicates on the one hand that we do not describe an artifact of the model we chose. On the other hand, it suggests a direct relationship between the concentration of pKi-67 and progression of the cell cycle [Scholzen and Gerdes, 2000]. Based on pKi-67's binding to the DNA synthesis controlling proteins MCM2 and p95 [Todorov et al., 1994; Varon et al., 1998; Schmidt et al., 2002], we speculate that the Ki-67 antigen is involved in the cellular decision to initiate DNA replication. Hampering its function results in less effective control and, therefore, in a faster passage through S-phase.

Apoptosis analysis of transiently transfected cells revealed that the cell cycle influencing pKi-67 fragments had a slight anti-apoptotic effect. Since we used PARP staining we possibly detected less apoptotic cells as a consequence of the reduced amount of cells in S-phase. Due to the diminished amount of cells in this phase, we measured less activation of PARP by DNA double strand breaks, possibly showing up as an anti-apoptotic effect in our model. Further investigations with different apoptosis analysis techniques (Annexin V staining, caspase assays) have to reveal, if we truly see an anti-apoptotic effect or a side-effect of pKi-67's influence on the cell cycle.

Finally, we investigated if non-proliferating cells express pKi-67 or not. It was previously described that cell cycle arrested cells can be MIB-1 positive [van Oijen et al., 1998]. MacCallum and Hall [2000] proposed, pKi-67 could act as a ribosomal catalyst, increasing the number of ribosomes and, therefore, the amount of protein production in the cell. Our group found pKi-67 to be critically involved in the lifecycle of the nucleolus itself, where ribosome generation is carried out [Schmidt et al., 2002]. Additionally, we found in this work a shift of cells from S to G_1/G_0-phase after expression of the pKi-67 C-terminus. We did not rule out the possibility that this construct could cause cells to differentiate and thereby enter G_0-phase. Therefore, it seemed reasonable that pKi-67 might be expressed in highly protein-synthesizing cells to enhance ribosome production. For that purpose we stained glandular and neuronal cells with MIB-1. In neither of the tissues we found any staining of the nucleus. Therefore, pKi-67 seems not to be transcribed in differentiated cells, even not as a ribosomal catalyst.

Summarizing, we found pKi-67 contributes to cell cycle regulation, maybe by effecting the control of DNA replication in S-phase due to its binding to DNA and the S-phase regulators MCM2 and p95. We found the level of pKi-67 to be self-regulated by reduction of its own transcription after expression of several of its own domains. Finally, we found a slight anti-apoptotic effect of recombinant pKi-67 fragments and we were able to underpin the restricted expression of the protein during the active phases of the cell cycle. This work opens the door for a more detailed analysis of the localization and function of pKi-67 within the complex cell cycle network.

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